EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Biosafety in the Laboratory

SOP Number: MB-01-04

Date Revised: 10-08-05

Initiated By: _____ Date: ___/___/___

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1.0 SCOPE AND APPLICATION:

1.1 This protocol outlines the required safety measures for working with the microorganisms received and maintained by the OPP Microbiology Laboratory.

1.2 The Laboratory takes a conservative approach towards working with microorganisms falling into the Biosafety Levels 1, 2 and 3. The availability of biological safety cabinets (BSCs) within the Laboratory, the ease and practicality of working within the BSC, and the ease of containing spills of chemical or biohazardous materials that occur within the BSC, provides a working environment in which all **manipulations** of cultures of microorganisms (section 2.6), regardless of the biosafety level, are done only in the BSC and not on the open bench.

1.3 This SOP is based largely on the guidance provided in the Centers for Disease Control and Prevention/National Institutes of Health (CDC/NIH) publication "Biosafety in Microbiological and Biomedical Laboratories," 4th ed. (BMBL; see ref. 15.3).

1.3.1 The Laboratory recognizes the biosafety levels set forth in the BMBL, and the need to provide different degrees of protection (i.e., ascending biosafety levels) depending upon the danger of the microbe to the worker, community, and the environment. The majority of the microorganisms contained in-house fall within Biosafety Levels 1 and 2.

1.3.2 This SOP is structured so all work involving manipulation of culture (section 2.6) of microorganisms is performed in the BSC.

1.3.2.1 In using a BSC for manipulation of all cultures, all microorganisms, regardless of biosafety level, are handled according to the following Biosafety Level 3 practices: A. Standard Microbiological Practices, B. Special Practices, and C. Safety Equipment (see ref. 15.3).

1.3.2.2 This SOP provides additional practices and procedures to be followed when working with Biosafety Level 3 microorganisms in order to provide analysts added protection from disease (see 10.0, Section II).

1.3.3 Recommendations set forth in Section D (Laboratory Facilities) of the BMBL's Biosafety Level 3 criteria apply only to Biosafety Level 3 microorganisms.

1.3.3.1 Manipulation (see section 2.6) of Biosafety Level 1 and 2 microorganisms may occur in either a Biosafety Level 2

(B201, B203, B204, B205, or B206) or a Biosafety Level 3 laboratory (B202, B207).

1.3.3.2 Manipulation (see section 2.6) of Biosafety Level 3 microorganisms may only occur in a Biosafety Level 3 laboratory (B202, B207).

1.4 Biological agents which are determined to be "Select Agents" (e.g., *Bacillus anthracis*; see ref. 15.4) and that are assigned to Biosafety Level 3, will be handled at a minimum according to Biosafety Level 3 practices. Additional personal protective equipment (PPE) and procedures will apply as specified in this SOP. See 10.0, Section III.

1.4.1 Currently, the only Select Agent addressed by this SOP is *Bacillus anthracis*. The SOP may be amended as necessary to address additional Select Agents.

1.5 Prior to manipulating (see section 2.6) cultures of a microorganism other than those listed in Attachment A, the laboratory staff must first determine the biosafety level of that microorganism (see Attachment A; see refs. 15.1 and 15.3).

1.6 The BMBL (see ref. 15.3) manual presents recommended guidelines for working with microorganisms assigned to Biosafety Levels 1 through 4. Although these guidelines are not currently legally enforceable guidelines, they are considered to be international standards of practice. Should an exposure event occur, the CDC/NIH guidelines could take on the force of law in that the laboratory management could be held legally responsible for not following accepted standards of practice (Dr. Robert McKinney, Director of the Division of Safety/NIH; personal communication to M. Cottrill, 7/10/98). Consequently, the laboratory will comply with the CDC/NIH guidelines.

2.0 DEFINITIONS:

2.1 *Mycobacterium bovis* (BCG) is a live attenuated vaccine strain used to vaccinate humans against infection by *Mycobacterium tuberculosis*.

2.2 BSC = biological safety cabinet

2.3 PPE = personal protective equipment

2.4 CDC = Centers for Disease Control and Prevention

2.5 NIH = National Institutes of Health

2.6 "Appropriate" disinfectant = EPA-registered hospital disinfectant (have label claims for *S. aureus*, *P. aeruginosa*, and *S. choleraesuis*) or hospital disinfectant with tuberculocidal claims (efficacious against *S. aureus*, *P. aeruginosa*, *S. choleraesuis*, and *M. bovis* (BCG)). All disinfectants must be used according to the directions (e.g., use dilution, contact time, etc.) specified on the labeling.

2.7 ATCC = American Type Culture Collection

2.8 OEP = Occupant Emergency Plan

2.9 CHP = Chemical Hygiene Plan

3.0 HEALTH AND SAFETY:

3.1 To protect the laboratory worker from possible infection by microorganisms, the health and safety guidelines provided in this protocol and in the BMBL (see ref. 15.3) manual must be followed. The manual is available in the laboratory. All laboratory personnel are required to read and familiarize themselves with the sections on Biosafety Levels 2 and 3.

3.2 Laboratory workers must familiarize themselves with this SOP, the laboratory's biosafety spill clean-up procedures (see SOP MB-13), and the facility's Chemical Hygiene Plan (CHP) prior to performing any laboratory work. Biosafety spill clean-up procedures are posted in the laboratories.

3.3 Laboratory workers are required to participate in the Agency's Occupational Medical Surveillance Program as established by EPA Order 1460.1. The Branch Chief evaluates the duties and responsibilities of the team and identifies the employees that are subject to exposure to chemical and biological agents in the laboratory. The names are forwarded to the ESC Safety, Health and Environmental Management (SHEM) manager who has responsibility for coordinating the medical monitoring program. The program is administered through the Department of Health and Human Services/U.S. Public Health Service.

3.4 Medical emergencies are handled according to procedures outlined in the ESC Occupant Emergency Plan (OEP). All emergencies are reported to the SHEM manager (or call security desk at extension 2800). The Branch Chief is responsible for documenting medical emergencies or accidents.

3.5 Spills and accidents are handled according to the practices outlined in this SOP and SOP MB-13, as well as procedures referenced in the OEP and Section 12 of the CHP. All spills and accidents are reported to the Branch Chief and the SHEM manager. The Branch Chief is responsible for documenting spills and accidents.

3.6 To promote the health of exposed individuals, the Branch Chief will encourage individuals to seek follow up, if necessary, depending upon recommendations of the SHEM manager.

3.7 All laboratory workers must meet the requirements of the Hazard Communication Program's Employee Training Program, as described in the CHP, section 8.

3.8 In accordance with the CDC/NIH guidelines (see ref. 15.3), the Branch Chief may restrict access to the laboratory as specified under "special practices".

3.9 All employees required to use respirators are participants in the Agency Occupational Medical Surveillance Program and the ESC Respiratory Protection Program. They have been medically cleared, fit tested for the specific respirator, and attended initial respirator use training. They receive annual respirator fit testing, annual use training, have documentation of training placed in the training file, and ensure that their respirators are inspected before and after each use, or at least monthly, (see 16.1) as specified in section 4 of the CHP.

3.10 No material suspected or known to be contaminated with biohazardous material (e.g., latex gloves, pipet wrappers, paper towels, etc.) is to be placed in the trash cans. These items are to be placed in an appropriate biohazardous waste bag (see section 7.3).

4.0 CAUTIONS:

4.1 Lack of following or understanding of this SOP may negatively impact the quality of the microbiological practices used in the laboratory and, hence, the laboratory's mission.

4.2 Failure to use the "STOP/DO NOT ENTER" signs to control access to the laboratory while cultures are being manipulated (see section 2.6) may result in the inadvertent exposure of personnel to biohazardous microorganisms.

4.3 Failure to clean the ultraviolet lamps in the BSCs will reduce the lamps' effectiveness. Periodically clean the ultraviolet lamps in the BSCs with a lint-free cloth dampened with alcohol (200 proof ethanol), and record the cleaning on the BSC Monitoring Record Form.

4.4 Autoclaving flasks containing diluted bleach may result in pitting of the autoclave's stainless steel interior.

4.5 Autoclaving flammable liquids (e.g., alcohols) is an explosion hazard. See 10.0, Section IV, 10.3 for guidance on autoclaving stain rinsate. Stain rinsate, although it contains low levels of ethanol and isopropanol, is sufficiently diluted in water

(>20% water) and is safe to autoclave (David Knower/Steris Corporation, personal communication to M. Cottrill, 5/23/03).

5.0 INTERFERENCES:

5.1 Failure to become familiar with and to put into practice the procedures set forth in this SOP will result in analysts who are a danger to themselves, others, and the environment.

5.2 Improper maintenance and/or sudden power failures may result in failure of the BSCs to operate properly. Refer to proper use and maintenance procedures in SOP QC-06, Use and Maintenance of Biological Safety Cabinets.

5.3 Proper certification of the BSCs must be maintained by the Facilities section.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

6.2 Each new analyst will complete twenty-four hours of initial safety training before entering the laboratory to work. All analysts will complete safety re-certification training (eight hours of training) on at least an annual basis. The facility SHEM manager is responsible for coordinating the training program.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Biological Safety Cabinet (BSC)

7.2 Autoclave

7.3 Biohazard bags (clear in color, autoclavable) or containers inside and outside of the biological safety cabinets for collection and storage of biohazardous waste.

7.4 When specified, personal protective equipment (PPE) such as latex gloves, latex inner glove/nitrile outer glove combination (for work with risk of exposure to *Bacillus anthracis*), safety glasses, lab coats, disposable tyvek laboratory garments, booties, and respiratory protection (P-100 half-face respirators with HEPA filter cartridges).

7.5 Appropriate signs to identify biohazardous materials and to limit access to laboratories.

7.6 Bleach solutions made fresh as needed. Discard waste solution appropriately at the end of the day.

7.6.1 Bleach solution to be used as a disinfectant to kill vegetative cells. Prepare a 1:10 dilution of an EPA-registered bleach product (containing at least 50,000-60,000 ppm sodium hypochlorite). Final concentration of sodium hypochlorite in the 1:10 bleach solution must be 5000-6000 ppm.

7.6.2 Bleach solution to be used as a sporicide to treat spore-forming bacteria. Prepare a 1:11.4 dilution of an EPA-registered bleach product (containing at least 50,000-60,000 ppm sodium hypochlorite) at approximately neutral pH in the following manner: 1 part EPA-registered household bleach product containing 50,000-60,000 ppm sodium hypochlorite: 9.4 parts water: 1 part distilled white vinegar (5% acetic acid).

7.6.2.1. The OPP Microbiology Laboratory has evaluated the efficacy of bleach as a sporicide using the AOAC Sporicidal Activity Test (modified). The Laboratory found that a 1:11.4 solution of bleach at approximately neutral pH with a contact time of 60 minutes is effective against 5 to 6 logs of *Bacillus subtilis* spores.

7.7 Appropriate disinfectants for microorganisms listed in Attachment A.

7.7.1 For microorganisms in vegetative form, use a 1:10 bleach solution as described in section 7.6.1, Lysol IC Brand Disinfectant Cleaner (EPA Reg. No. 675-43) at a 1:200 dilution (5 mL disinfectant + 995 mL water), or other EPA- registered hospital disinfectant/tuberculocide.

7.7.2 For microorganisms in spore form, prepare the adjusted bleach solution described in section 7.6.2.

7.8 Key card readers are used to limit access to testing laboratories. Only authorized personnel are permitted to enter.

7.9 Bacteria maintained by the OPP Microbiology Laboratory for the Antimicrobial Product Testing Program and Quality Control of the VITEK 32 Automated Identification System (see SOP QC-17, VITEK: Quality Control) are specified in Attachment A.

8.0 INSTRUMENT OR METHOD CALIBRATION: Not applicable

9.0 SAMPLE HANDLING AND STORAGE: Not applicable

10.0 PROCEDURE AND ANALYSIS:

Section I. Procedures for Working with Biosafety Level 1 and 2 Microorganisms (see Attachment A).

10.1 **Access to Laboratories**.

10.1.1 Key card readers are used to limit access to testing laboratories. Only authorized personnel (e.g., laboratory staff, maintenance staff, etc.) are permitted to enter.

10.1.2. Further limit access to the laboratory when manipulating infectious microorganisms (see section 2.6) by posting the magnetic "STOP/DO NOT ENTER" sign on the outside (i.e., side facing corridor) of the external laboratory door.

10.1.3 Only laboratory staff are authorized to enter the laboratory while the STOP/DO NOT ENTER sign is posted.

10.1.4 Remove the "STOP/DO NOT ENTER" sign once work (section 2.6) is complete.

10.2 **Personal Protective Equipment (PPE).**

10.2.1 BSCs.

10.2.1.1 If ultraviolet light was left on overnight for decontamination purposes, turn it off.

10.2.1.2 Turn on the blowers, lights, and outlets, and allow to operate for a minimum of 15 minutes before aseptic manipulations are begun in the cabinet.

10.2.1.3 Disinfect BSC surface prior to use (see 10.0, Section I, 10.4.1).

10.2.1.4 Record the Downflow (FPM) and Exhaust (CFM) rates on the BSC Monitoring Record Form immediately prior to use (see SOP QC-06, Use and Maintenance of Biological Safety Cabinets).

10.2.1.5 See SOP QC-06, Use and Maintenance of Biological Safety Cabinets, for more details.

10.2.2 Safety glasses. Safety glasses must be worn while working in the laboratory. Safety glasses do not have to be worn while doing paperwork

in the laboratory or when entering the laboratory solely to retrieve an item such as a document, *provided that no manipulation of cultures (see section 2.6) or other laboratory work is in progress*. However, safety glasses should be immediately available in the work area.

10.2.3 Lab coats. Wear cloth lab coats while working in the laboratory with Biosafety Level 1 and 2 microorganisms.

10.2.3.1 Lab coats do not have to be worn while doing paperwork in the laboratory or when entering the laboratory solely to retrieve an item such as a document, *provided that no manipulation of cultures (see section 2.6) or other laboratory work is in progress*.

10.2.3.2 Remove lab coats before going to non-laboratory areas such as the office areas, restrooms, cafeteria, library, etc.

10.2.4 Gloves. Wear gloves (latex or nitrile) when manipulating culture (see section 2.6) and when handling any vessel (e.g., test tube rack, test tube, plate, biohazard bag), closed or open, containing live organism.

10.2.4.1 Prior to beginning work, inspect gloves. Do not use gloves that have holes, rips, or are otherwise degraded.

10.2.4.2 Replace gloves immediately in the event of overt contamination with infectious material. Dispose of contaminated gloves in the biohazard bin only.

10.2.5 Shoe covers/booties. Not required for work with Biosafety Level 1 or 2 microorganisms.

10.2.6 Face protection. If a certain procedure involving manipulation of the organism is impossible or impractical to conduct within the BSC (e.g., reading the percent transmittance of a culture), face protection (e.g., a face splash shield) must be used for protection from anticipated or unanticipated splashes or sprays of infectious materials to the face.

10.2.6.1 Transporting closed petri dishes (containing seeded carriers) to the incubator for drying and counting colonies on plates which are closed and wrapped with parafilm are not considered to be manipulation of culture.

10.2.7 Respiratory Protection. Respirators are not required for manipulation of Biosafety level 1 and 2 microorganisms.

10.3 **Activities Required to be Performed in the BSC.** Specifically, conduct the following manipulations in the BSC:

10.3.1 Handling any open vessel or plate containing microorganism

10.3.2 Conducting culture transfers

10.3.3 Plating and spread-plating activities

10.3.4 Pipetting culture

10.3.5 Harvesting culture

10.3.6 Seeding carriers

10.3.7 Dropping carriers into disinfectant/neutralizer/subculture media tubes

10.3.8 Vortexing/sonicating tubes containing live microorganisms

10.3.9 Reading results from tubes and plates (unless plates are closed and wrapped with parafilm)

10.3.10 Smear preparation (microscope slides)

10.4 **Treatment of Equipment Post-Use.**

10.4.1 BSCs. BSC surfaces must be disinfected prior to and after working with infectious material and immediately after any spill of infectious material. Spray the surface of the BSC with the use dilution of an appropriate disinfectant (e.g., Lysol IC Brand Disinfectant Cleaner 1:200, see section 7.7.1). Allow the surface to remain wet for the label-specified contact time. Halogenated materials are not recommended for routine use on stainless steel surfaces of the BSC

10.4.1.1 As an additional step, at the conclusion of activities involving bacteria in spore form (e.g., *Bacillus subtilis* spore suspensions, spore strips), turn on the ultraviolet light and leave it on overnight. Visually verify that UV bulb is clean. If not, wipe with alcohol and record in BSC Monitoring Record Book.

10.4.2 Chillers. On a weekly basis, following testing, disinfect the water in the recirculating chiller and remote water bath prior to draining.

10.4.2.1 Disinfect the water by adding the appropriate amount of a disinfectant labeled for use against the test organisms (e.g., Lysol I.C Brand Disinfectant Cleaner, EPA Reg. No. 675-43, 1:200 dilution) to the recirculating chiller and remote water bath. Follow label directions for use. Record information on the Recirculating Chiller Cleaning and Disinfection Log (see 16.0).

10.4.2.2 During use, the recirculating chiller/remote waterbath system circulates approximately 23.5 L of water (20.5 L in chiller reservoir + 3 L in remote waterbath). To achieve a 1:200 dilution of Lysol IC Brand Disinfectant Cleaner in the circulating water, add 119 mL disinfectant to the water in the chiller reservoir or to the remote water bath.

10.4.2.3 After the disinfectant is added, the unit should be turned on and run thoroughly. Turn off the unit and allow the disinfectant to remain in the unit for the specified contact time (e.g., 10 minutes).

10.4.2.4 Each recirculating chiller is equipped with a drain valve and a drain hose located on the back of the unit. With the unit off, open the valve and allow the reservoir contents to drain into an appropriately sized container or directly into a sink.

10.4.2.5 Rinse the unit by filling it with tap water. Allow it to run for approximately 10 minutes. Turn off the unit and drain as above. Refill the reservoir with fresh tap water on the day of testing.

10.4.3 Sonicator. If the sonicator has been used in a given week, disinfect the water in the sonicator bath, at the end of the testing week, by adding appropriate disinfectant to the water in the bath to achieve the disinfectant product's use dilution. Let the disinfectant remain in the sonicator bath for the contact time stated on the disinfectant labeling. Once the contact time is achieved, discharge the treated water appropriately, rinse the unit with tap water, and dry the sonicator bath with paper towels.

10.4.3.1 For Lysol IC Brand Disinfectant Cleaner (EPA Reg. No. 675-43), use a 1:200 dilution (7.6 mL disinfectant added to 1500 mL water in sonicator) for a contact time of ten minutes.

10.4.4 Spectrophotometer. After each use of the spectrophotometer, remove the cell holder from the instrument and disinfect it with an appropriate disinfectant (e.g., Lysol IC Brand Disinfectant Cleaner 1:200, see section 7.7.1). Allow the surface to remain wet for the label-specified contact time. Thoroughly rinse with water, allow to dry, and replace cell holder in the spectrophotometer.

10.4.4.1 Disinfectant is not to be sprayed or wiped on the inner surfaces of the spectrophotometer as disinfectant residue may remain on the optics, negatively impacting instrument operation.

10.5 **Staining Procedures.**

10.5.1 While staining and viewing slides, wear gloves and a lab coat, and conduct any steps involving manipulation of the organism (e.g., smear preparation) in the BSC.

10.5.2 Decontamination of rinsate.

10.5.2.1 Collect the rinsate and add household bleach (EPA-registered bleach product containing at least 50,000-60,000 ppm sodium hypochlorite) full strength to the rinsate in an approximate 1:10 ratio (one part household bleach to nine parts rinsate) (see ref. 15.1, pg. 390) for a minimum of 60 minutes (in the event that the rinsate contains spores) before disposal.

OR

10.5.2.2 Collect rinsate and autoclave as specified in 10.0, Section IV, 10.3.

10.5.3 After microscopically viewing organisms, remove slides from the microscope stage and discard them in a biohazard bin. If it is necessary or desirable to keep a prepared slide, store it in a sealed petri dish or a microscope slide case to which a biohazard label has been affixed.

10.6 **Biohazardous Waste.** After manipulating culture (see section 2.6), analysts must bag biohazardous waste and place it in a closed container (e.g., biohazard bin with lid, biohazard bag taped shut).

10.6.1 See 10.0, Section IV for autoclaving of biohazardous waste.

10.6.2 Biohazardous waste may not be moved outside of the second floor B-wing prior to sterilization.

10.7 **Transport of Cultures.**

10.7.1 When removing live cultures (e.g., agar plates, racks of tubes, biohazard bags containing biohazardous waste) from the immediate laboratory for incubation in other laboratories or for decontamination purposes, place the cultures in durable, leak-proof containers (e.g., Nalgene tub) for transport from the laboratory.

10.7.2 Autoclave bags containing biohazardous waste should be taped shut prior to transport.

10.7.3 Do not transport the live cultures outside of the microbiology laboratory wing.

10.8 **Always Wash Hands Prior to Exiting Laboratory.**

Section II. Procedures for Working with Biosafety Level 3 Microorganisms (Excludes Select Agents; see Attachment A).

10.1 Follow the procedures outlined for working with Biosafety Levels 1 and 2 microorganisms (10.0, Section I) with the following exceptions/additions:

10.2 All procedures involving *M. bovis* (BCG) or any other Biosafety Level 3 microorganism must be performed in a Biosafety Level 3 laboratory (Rooms B202 and B207).

10.3 **Personal Protective Equipment (PPE).**

10.3.1 Disposable lab coats. Wear disposable lab coats while working in the Biosafety Level 3 laboratory. Cloth lab coats may not be worn in the Biosafety Level 3 laboratory during culture manipulation procedures (section 2.6).

10.3.1.1 Lab coats do not have to be worn while doing paperwork in the laboratory or when entering the laboratory solely to retrieve an item such as a document, *provided that no manipulation of cultures (see section 2.6) or other laboratory work is in progress.*

10.3.1.2 Replace disposable lab coat immediately in the event of suspected or known contamination with infectious material. Dispose of contaminated lab coat in a biohazard bin.

10.3.1.3 All disposable lab coats worn in the laboratory when cultures are being manipulated may **not** be worn out of the Biosafety Level 3 laboratory. This includes lab coats worn by personnel who are present in the laboratory but not directly involved in culture manipulation. Prior to exiting the laboratory and entering the double door access zone, remove and discard lab coats (in biohazard bin) according to the descriptions of activities provided in sections 10.3.1.4 and 10.3.1.5.

10.3.1.4 Disposable lab coats worn while harvesting or homogenizing cultures of a Biosafety Level 3 microorganism must be discarded in a biohazard bin before the analyst leaves the laboratory and enters the double-door access zone. The lab coat may not be stored in the laboratory for re-use later in the day.

10.3.1.5 Disposable lab coats worn for purposes other than harvesting or homogenizing cultures of a Biosafety Level 3 microorganism during the day may be stored in a designated area in the laboratory in the event that the analyst leaves the laboratory (e.g., to retrieve an item or take a break). Upon return, the analyst may re-use the lab coat to resume laboratory activities. At the end of each day, all used disposable lab coats must be disposed of in the biohazard bin.

10.3.2 Gloves.

10.3.2.1 Wear a single pair of gloves (latex or nitrile) when manipulating culture (see section 2.6). Prior to leaving the BSC to conduct other activities (e.g., open the incubator, record data, retrieve supplies, etc.), discard the gloves in the biohazard bin. Put on a new pair of gloves upon returning to the BSC or before handling any vessel containing live organism.

OR

10.3.2.2 Wear a double layer of gloves (latex or nitrile) when manipulating culture (see section 2.6). Prior to leaving the BSC to conduct other activities (e.g., open the incubator, record data, retrieve supplies, etc.), the outer pair of gloves

must be discarded in the biohazard bin. Replace the outer gloves upon returning to the BSC.

10.3.3 Shoe covers/booties. Wear shoe covers or tyvek booties when manipulating culture in the Biosafety Level 3 laboratory (see section 2.6).

10.3.3.1 Remove shoe covers or booties prior to exiting the laboratory and discard in the biohazard bin. Do not wear shoe covers or booties into the double door access room.

10.3.4 Respiratory protection. If a certain procedure involving manipulation of the organism is impossible or impractical to conduct within the BSC (e.g., reading the percent transmittance of a culture), respiratory protection (P-100 half-face respirators with HEPA filter cartridges) must be worn while working with the organism outside of the BSC.

10.3.4.1 Transporting closed petri dishes (containing seeded carriers) to the incubator for drying and counting colonies on plates which are closed and wrapped with parafilm are not considered to be manipulation of culture. Wearing respiratory protection is not mandatory for these two activities.

10.4 **Equipment.**

10.4.1 Administrative supplies. Ink pens and clip boards used to record data while manipulating cultures (see section 2.6) will be identified for use in the Biosafety Level 3 laboratories. These items are not to leave the BSL 3 labs.

10.4.1.1 Discard all old or broken items in a biohazard bag.

10.5 **Transport of Cultures.** Remove and discard disposable lab coat and gloves (latex or nitrile), and shoe covers/booties prior to exiting the Biosafety Level 3 laboratory and replace them with a clean disposable coat and a new set of gloves (latex or nitrile) to be worn during the transport process.

Section III. Procedures for Working with Environmental Samples and Spore Strips with Potential Risk of Exposure to the Select Agent *Bacillus anthracis*.

10.1 **Sample Receipt.** Upon delivery to the Environmental Science Center, the samples will be handled in room D122.

10.2 All analyses with potentially-contaminated samples will be conducted in a Biosafety Level (BSL) 3 laboratory.

10.3 All lab work will be conducted, at a minimum, according to BSL 3 practices (see 10.0, Section II). See additional Personal Protective Equipment (PPE) and procedures below.

10.4 **Required PPE.** Analysts must wear the following PPE when conducting any work with potential risk of exposure:

10.4.1 Tyvek suits

10.4.2 Double layer of gloves (inner layer-latex, outer layer- nitrile)

10.4.3 Booties if tyvek suits do not have feet

10.4.4 P-100 half-face respirators with HEPA filter cartridges.

10.4.5 Safety glasses

10.5 **PPE Donning Procedures.**

10.5.1 Put on tyvek suit and booties.

10.5.2 Tape any loose-fitting clothing.

10.5.3 Double glove (latex inner, nitrile outer).

10.5.4 Pull cuff of outer, nitrile glove over the sleeve of the tyvek suit.

10.5.5 Have co-worker place a piece of tape (preferably duct tape) around your wrist, taping the outer glove to the tyvek suit. Turn the tape over on itself at the end in order to create a tab for easy tape removal.

10.5.6 Put on a sleeve over the tyvek suit, covering the wrist area.

10.5.7 Put on respirator with **new** HEPA (P-100) cartridges.

10.5.8 Put on safety glasses.

10.6 **PPE Doffing Procedures.**

10.6.1 With hands and forearms still in the biosafety cabinet (BSC), remove the sleeve, inverting it as it is removed. Discard the sleeve in a biohazard bag that has been placed in the BSC.

10.6.2 Grabbing the tape tabs, peel off the tape holding the outer glove to the tyvek suit, while taking care not to remove the inner glove. Discard tape in the biohazard bag in the BSC.

10.6.3 Remove the first outer glove by pinching the glove near the cuff with the thumb and forefinger of the opposite hand and pulling the glove off, inverting it. While still holding the first outer glove, repeat the process with the second outer glove, inverting the second glove over top of the first glove during the removal process. Take care not to remove the inner glove. Discard gloves in biohazard bag in the BSC.

10.6.4 Close biohazard bag in the BSC and place it in biohazard bag outside of the BSC.

10.6.5 While still wearing the inner layer of gloves and a respirator, remove the tyvek suit and then the shoe covers, and place them in the large biohazard bag outside of the BSC.

10.6.6 Remove the inner layer of gloves by inverting them as above, then place them in the large biohazard bag outside of the BSC.

10.6.7 Put on a new set of gloves (latex or nitrile), take off the respirator, and place it in the BSC. Remove the two respirator filter cartridges and discard them in the biohazard bag outside of the BSC.

10.6.8 While still wearing gloves, carry the respirator to the sink and wash it thoroughly with mild soap and water. Rinse respirator with water. Allow to dry before storing in plastic storage bag.

10.6.9 Discard gloves in biohazard bag outside of the BSC.

10.7 **Additional Procedures.**

10.7.1 Buddy System Requirement. At least two analysts with specified PPE will be present in the lab during all work.

10.7.2 Staining.

10.7.2.1 Stain preparation (i.e., smears, heat fixing) will be conducted in the BSC.

10.7.2.2 Slides will be read using a microscope in the BSL 3 lab.

10.7.2.3 After reading, slides will be decontaminated by soaking in bleach solution (see section 7.6.2) for one hour or by autoclaving as specified in 10.0, Section III, 10.7.4 and 10.0, Section IV.

10.7.2.4 Rinsate will be collected and autoclaved as specified in 10.0, Section III, 10.7.4 and 10.0, Section IV, 10.3.

10.7.3 Decontamination of instruments. Instruments (e.g., forceps) used will be decontaminated by a 60-minute exposure to a 1:11.4 bleach solution at approximately neutral pH (see section 7.6.2) or by autoclaving as specified in 10.0, Section III, 10.7.4 and 10.0, Section IV.

10.7.4 Decontamination of biohazardous waste.

10.7.4.1 Biohazardous waste generated from the study will be autoclaved daily using a 3 hour liquid kill cycle.

10.7.4.2 In addition to the maximum registering thermometer and chemical strip, a biological spore ampule will be included with each run to provide additional quality control. While wearing PPE (section III, 10.4, Required PPE), place a biological indicator ampule in the center of an autoclave bag containing solid biohazardous waste or submerged in a tube/flask if autoclaving liquids. It may be useful to tie a string to the ampule prior to placing it in the bag or flask for ease of retrieval after cycle completion. Remove PPE as in section III, 10.6, and place it in the autoclave bag. Put on a disposable lab coat, booties, and two layers of gloves to handle autoclave bag and place it in the autoclave. Dispose disposable lab coat, booties, and gloves in another autoclave bag. This bag may be treated in a future kill cycle, as the risk of select agent contamination of this set of PPE is low.

10.7.4.3 After completion of the cycle, verify performance of the autoclave using the autoclave print out (i.e., verify minimum temperature was at least 121°C), maximum registering thermometer (i.e., reads at least 121°C), and chemical indicator strip (passing result) (see SOP QC-13, Performance Verification of Autoclaves). If performance of the autoclave is acceptable, don gloves and cloth labcoat to retrieve the ampule. Label the ampule and incubate it for 48 hours at 55-60°C along with a control ampule that has not been autoclaved. Record results on the Daily

Sterilization Record Information Log Form (see SOP QC-13, Performance Verification of Autoclaves).

10.7.4.4 If any of the three performance verification parameters (autoclave print out, maximum registering thermometer, or chemical indicator strip) indicates that the autoclave run was unsuccessful, the biohazardous waste must be re-autoclaved. Do not retrieve the biological ampule prior to repeating the autoclave cycle.

10.7.4.5 Do not dispose of autoclaved biohazardous waste until incubation of the biological spore ampule is complete and results indicate that all spores in the ampule were killed during the autoclave cycle. Close or cover the biohazard bag or flask and store it in a bin inside the laboratory. Label the material in some way to ensure that it is not confused with unautoclaved material.

10.7.5 BSC cleanup.

10.7.5.1 For cleanup of BSC after use, wipe or spray surface with of an EPA-registered hospital disinfectant (e.g., Lysol IC Brand Disinfectant Cleaner 1:200, see section 7.7.1) to physically remove spores. Hospital disinfectants are not sporicidal.

10.7.5.2 Allow the surface to remain wet for the label-specified contact time, and then dry the surface with paper towels.

10.7.5.3 Dispose of paper towels by autoclaving as specified in 10.0, Section III, 10.7.4 and 10.0, Section IV.

10.7.5.4 To destroy live spores remaining on the surfaces of the BSC, turn on the ultraviolet (UV) light in the BSC and leave it on overnight (minimum of 15 hours).

10.7.5.4.1 Record the use of the UV light, the time the UV light is turned on, and the time it is turned off in the BSC Monitoring Record Book.

10.7.6 Respirator cleanup.

10.7.6.1 Remove filter cartridges from respirator and place in a biohazardous waste bag and decontaminate by autoclaving as specified in 10.0, Section III, 10.7.4 and 10.0, Section IV.

10.7.6.2 Wash respirator with mild soap and water. Thoroughly rinse with water.

10.7.6.3 Once clean and dry, store respirator in a plastic bag.

10.7.7 Transport/storage of culture tubes.

10.7.7.1 Secondary containment (Nalgene tub) will be used for transport and storage of culture tubes within the Biosafety Level 3 laboratory.

10.7.7.2 No samples, cultures, slides, etc. will be moved from the Biosafety Level 3 laboratory prior to being autoclaved.

Section IV. Autoclaving Biohazardous Waste Materials.

10.1 Storage of Items Awaiting Sterilization.

10.1.1 No biohazardous waste may be removed from the second floor B-wing prior to sterilization.

10.1.2 Use autoclave bins. Some procedures result in the constant generation of contaminated articles that must be removed from the BSC work area and stock-piled on a countertop or table for autoclaving at the completion of the procedure or the next morning. In this case, place all contaminated articles in autoclavable bins.

10.1.2.1 Small items. Place contaminated cuvettes, homogenizers, and other small equipment into a beaker covered with aluminum foil prior to placing the items in the autoclavable bin.

10.1.3 Close containers. Keep all biohazardous waste-containing articles (e.g., autoclave bags, containers, tubes, flasks, homogenizers, cuvettes, etc.) closed, covered, or in the BSC while awaiting sterilization in order to prevent the generation and release of infectious aerosols into the laboratory environment.

10.1.3.1 Cover liquid waste with foil. All test tubes/flasks containing liquid waste (including used micropipette tips) must be capped or covered with aluminum foil.

10.1.3.2 Autoclave bags. Tape full autoclave bags closed.

10.2 **Preparation of Autoclave Bags.**

10.2.1 To prepare autoclave bags of biohazardous waste for sterilization, place one bag in an autoclavable bin.

10.2.2 Open the bag and pour approximately 250 mL of water into the bag and 250 mL of water into the bin.

10.2.3 Make sure that the bag is opened wide prior to placing the bin into the autoclave.

10.3 **Preparation of Containers of Liquid Waste and Small Items.**

10.3.1 To prepare containers of liquid waste and materials such as contaminated micropipette tips, homogenizers, racks, cuvettes, and glassware for autoclaving, place the items in an autoclavable bin.

10.3.2 Add approximately 250 mL of water to the bin.

10.3.3 Place the bin into the autoclave.

10.3.4 No liquid waste containing bleach (will damage stainless steel interior of autoclave) or flammable liquid (explosion hazard) is to be autoclaved.

10.3.4.1 Up to a liter of stain rinsate may be safely autoclaved. Rinsate contains low levels of isopropanol and ethanol. However, these flammable components are sufficiently diluted in water (>20% water), rendering the rinsate safe for autoclaving (David Knower/Steris Corporation, personal communication to M. Cottrill, 5/23/03).

10.4 Use a **three hour** (180 minute) **liquid cycle** to sterilize both liquid and solid biohazardous waste.

10.5 See QC-13, Performance Verification of Autoclaves, for verification of autoclave performance and corrective actions.

10.6 Resource Management.

10.6.1 Water Conservation. Laboratory personnel should be mindful of water consumption, and whenever possible, employ practices that minimize water use.

10.6.1.1 Specifically, laboratory personnel should run full autoclave loads whenever possible.

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Results of the monthly respirator check will be recorded legibly and in indelible ink on the Respirator Inspection Checklist form (see 16.0). The forms will be kept in the Respirator Inspection Notebook.

12.2 The Respirator Inspection Notebook is kept in room B202 rather than the file room, D217. In addition to monthly inspections, respirators must be inspected prior to each use. Therefore, it is crucial that the Respirator Inspection Notebook be readily available rather than retained in a file room. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives. Copies of the Respirator Inspection Checklist forms are presented to the SHEM manager during annual respirator fit-testing.

12.3 Data will be recorded promptly, legibly, and in indelible ink on the BSC Monitoring Record Form (see SOP QC-06, Use and Maintenance of Biological Safety Cabinets) and the Sonicator Disinfection Log. Completed forms are archived in notebooks kept in secure file cabinets in file room D217. Only authorized personnel have access to the secure files. Archived data are subject to OPP's official retention schedule contained in SOP ADM-03 (Records and Archives).

12.4 The Branch Chief is responsible for documenting medical emergencies, accidents, and spills.

13.0 QUALITY CONTROL: None

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Strict adherence to the biosafety practices is required. Nonconformance will result in notification, retraining, or possible disciplinary action of laboratory employees.

15.0 REFERENCES:

15.1 Fleming, D.O. and Hunt, D.L. eds. 2000. Biological Safety: Principles and Practices. ASM Press, Washington, D.C.

15.2 Official Methods of Analysis. 1995. 16th Ed. AOAC INTERNATIONAL, Gaithersburg, MD. Chapter 6, Subchapter 2, entitled "Hard Surface Carrier Test Methods.

15.3 Richmond, J.Y. and McKinney, R.W. eds. 1999. Biosafety in Microbiological and Biomedical Laboratories. HHS Publication No. (CDC) 93-8395. U.S. Government Printing Office, Washington, D.C.

15.4 Additional Requirements for Facilities Transferring or Receiving Select Agents, 42 CFR Part 72.6.

15.5 The CDC/NIH manual assigns propagation and manipulation of cultures of *Mycobacterium tuberculosis* and *Mycobacterium bovis* (BCG) to Biosafety Level 3. The manual does not specifically address propagation and manipulation of cultures of *M. bovis* (BCG). NIH recommends that laboratories follow Biosafety Level 3 guidelines when handling *M. bovis* (BCG) (Dr. Robert McKinney, personal communication to M. Cottrill, 7/23/96). Those laboratories unable to meet the Biosafety Level 3 facility requirements may, at a minimum, handle cultures of *M. bovis* (BCG) in a Biosafety Level 2 facility while employing Biosafety Level 3 practices.

M. bovis (BCG) is as transmissible to humans via the aerosol route as *M. bovis.* If a laboratory worker seroconverts (i.e., has a positive tuberculosis skin test), it is impossible to determine with absolute certainty that the seroconversion was due to exposure to *M. bovis* (BCG) and not *M. tuberculosis*. Consequently, chest x-rays and drug therapy may become necessary to ensure that the worker does not have an active tuberculosis infection and is not infectious.

The EPA/OPP Microbiology Laboratory will comply with Biosafety Level 3 guidelines (Sections A, B, C, and D) when handling *M. bovis* (BCG).

16.0 FORMS AND DATA SHEETS:

16.1 Respirator Inspection Checklist.

16.2 Recirculating Chiller Cleaning and Disinfection Log.

16.3 Sonicator Disinfection Log.

Attachment A: Bacteria Maintained by the OPP Microbiology Laboratory.

Respirator Inspection Checklist

OPP Microbiology Laboratory

Respirators must be inspected before and after each use, and monthly. A respirator kept ready for emergency use must be inspected before and after each use and monthly to assure that it is maintained in satisfactory condition. Use this sheet to document the inspections.

Name	
Type of Respirator	
Storage Location	

Date										
Facepiece										
Connections										
Headbands										
Valves										
Cartridges										
Other										
Comments										

Safety Office Review: _____ Date: _____

Recirculating Chiller Cleaning and Disinfection Log
OPP Microbiology Laboratory

INFORMATION			
Chiller #	Location	Date/Init.	Action*

* Record the action performed, i.e. disinfected, drained, cleaned, etc.

Sonicator Disinfection Log

OPP Microbiology Laboratory

INFORMATION				
Date/Init.	Unit (check a box)		Use	Treatment Performed (product, dilution, contact time)*
	Serial Number RKA10980 258D	Serial Number RKC1002 74019D		

* Units hold approximately 1500 mL of water. For Lysol IC Brand Disinfectant Cleaner (EPA Reg. No. 675-43), use a 1:200 dilution (7.6 mL disinfectant added to 1500 mL water in sonicator) for a contact time of ten minutes.

Attachment A:

Bacteria Maintained by the OPP Microbiology Laboratory for the Antimicrobial Product Testing Program and Quality Control of the VITEK 32 Automated Identification System.
OPP Microbiology Laboratory

See next page.

Organism	ATCC #	Biosafety Level
Mycobacterium bovis (BCG)*	Not applicable	3[++]
*Pseudomonas aeruginosa**	15442	2[+]
Pseudomonas aeruginosa (Schroeter) Migula****	47085	2
*Staphylococcus aureus**	6538	2[+]
*Bacillus licheniformis***	12759	1
*Bacillus sphaericus***	4525	1
*Bacillus subtilis**	19659	1
*Clostridium sporogenes*****	3584	1
*Serratia liquefaciens***	27592	1
*Leclercia adecarboxylata***	23216	1
*Burkholderia cepacia***	25608	1
*Enterococcus durans***	6056	1
*Streptococcus bovis***	9809	1
*Enterococcus faecalis***	29212	1
*Escherichia coli DH5 (Host)*****	87482	1
*Escherichia coli DH5 (Host)*****	87483	1
*Escherichia coli DH5 (Host)*****	87484	1
*Escherichia coli DH5 (Host)*****	87485	1
*Proteus mirabilis***	7002	2
*Providencia alcalifaciens***	51902	2
*Klebsiella pneumoniae***	13883	2
*Plesiomonas shigelloides***	51903	2
*Bordetella bronchiseptica***	10580	2
*Streptococcus equi***	9528	2
*Erysipelothrix rhusiopathiae***	19414	2
*Streptococcus pyogenes***	19615	2
*Staphylococcus xylosus***	29971	2

*Used as a Test Microbe in the Antimicrobial Testing Program
**Used in Quality Control of the VITEK 32 Automated Identification System
***Potentially to be used as a control organism for presumptive testing of remediation samples
**** Used only by Dr. Freshteh Toghrol (Senior Scientist, Biological and Economic Analysis Division) and her staff. **Dr. Toghrol's laboratory and biological safety cabinet are of limited size. Therefore, she or her staff may periodically use OPP Microbiology Laboratory space (manipulate microorganisms in BSC #1 and utilize incubator #4 in room B204).**
*****For use in sporicidal method-related research.**
[+] See reference 15.2.
[++] See reference 15.5.